



Published in final edited form as:

*Genet Med.* 2020 December ; 22(12): 2020–2028. doi:10.1038/s41436-020-0908-0.

## Likely damaging *de novo* variants in congenital diaphragmatic hernia patients are associated with worse clinical outcomes

Lu Qiao, PhD<sup>1,2</sup>, Julia Wynn, MS<sup>1</sup>, Lan Yu, PhD<sup>1</sup>, Rebecca Hernan, MS<sup>1</sup>, Xueya Zhou, PhD<sup>1,2</sup>, Vincent Duron, MD<sup>3</sup>, Gudrun Aspelund, MD MS<sup>3</sup>, Christiana Farkouh-Karoleski, MD<sup>1</sup>, Annette Zygumunt, PhD<sup>1</sup>, Usha S. Krishnan, MD<sup>1</sup>, Shannon Nees, MD<sup>1</sup>, Julie Khlevner, MD<sup>1</sup>, Foong Yen Lim, MD<sup>4</sup>, Timothy Crombleholme, MD<sup>4</sup>, Robert Cusick, MD<sup>5</sup>, Kenneth Azarow, MD<sup>6</sup>, Melissa Ellen Danko, MD<sup>7</sup>, Dai Chung, MD<sup>7</sup>, Brad W. Warner, MD<sup>8</sup>, George B. Mychaliska, MD MS<sup>9</sup>, Douglas Potoka, MD<sup>10</sup>, Amy J. Wagner, MD<sup>11</sup>, Samuel Soffer, MD<sup>12</sup>, David Schindel, MD<sup>13</sup>, David J. McCulley, MD<sup>14</sup>, Yufeng Shen, PhD<sup>2,15,16,#</sup>, Wendy K. Chung, MD PhD<sup>1,17,18,#</sup>

<sup>1</sup>Department of Pediatrics, Columbia University Irving Medical Center, New York, NY, USA

<sup>2</sup>Department of Systems Biology, Columbia University Irving Medical Center, New York, NY, USA

<sup>3</sup>Department of Surgery, Columbia University Irving Medical Center, New York, NY, USA

<sup>4</sup>Cincinnati Children's Hospital, Cincinnati, OH, USA

<sup>5</sup>Children's Hospital & Medical Center of Omaha, University of Nebraska College of Medicine, Omaha, NE, USA

<sup>6</sup>Department of Surgery, Oregon Health & Science University, Portland, OR, USA

<sup>7</sup>Monroe Carell Jr. Children's Hospital, Vanderbilt University Medical Center, Nashville, TN, USA

<sup>8</sup>Washington University, St. Louis Children's Hospital, St. Louis, MO, USA

<sup>9</sup>Division of Pediatric Surgery, Department of Surgery, University of Michigan School of Medicine, Ann Arbor, MI, USA

<sup>10</sup>Division of Pediatric Surgery, Department of Surgery, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

<sup>11</sup>Medical College of Wisconsin, Milwaukee, WI, USA

<sup>12</sup>Department of Surgery, Northwell Health, Great Neck, NY, USA

<sup>13</sup>Children's Health Dallas, UT Southwestern Medical Center, Dallas, TX, USA

<sup>14</sup>Department of Pediatrics, University of Wisconsin-Madison, Madison, WI, USA

<sup>15</sup>Department of Biomedical Informatics, Columbia University Irving Medical Center, New York, NY, USA

Users may view, print, copy, and download text and data-mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use:[http://www.nature.com/authors/editorial\\_policies/license.html#terms](http://www.nature.com/authors/editorial_policies/license.html#terms)

# Correspondence should be addressed to Y.S. (ys2411@cumc.columbia.edu) and W.K.C. (wkc15@cumc.columbia.edu).

Conflict of interest

All authors declare no conflicts of interest.

<sup>16</sup>JP Sulzberger Columbia Genome Center, Columbia University Irving Medical Center, New York, NY, USA

<sup>17</sup>Department of Medicine, Columbia University Irving Medical Center, New York, NY, USA

<sup>18</sup>Herbert Irving Comprehensive Cancer Center, Columbia University Irving Medical Center, New York, NY, USA

## Abstract

**Purpose**—Congenital diaphragmatic hernia (CDH) is associated with significant mortality and long-term morbidity in some but not all individuals. We hypothesize monogenic factors that cause CDH are likely to have pleiotropic effects and be associated with worse clinical outcomes.

**Methods**—We enrolled and prospectively followed 647 newborns with CDH and performed genomic sequencing on 462 trios to identify *de novo* variants. We grouped cases into those with and without likely damaging (LD) variants and systematically assessed CDH clinical outcomes between the genetic groups.

**Results**—Complex cases with additional congenital anomalies had higher mortality than isolated cases ( $P=8\times 10^{-6}$ ). Isolated cases with LD variants had similar mortality to complex cases and much higher mortality than isolated cases without LD ( $P=3\times 10^{-3}$ ). The trend was similar with pulmonary hypertension at 1 month. Cases with LD variants had an estimated 12–17 points lower scores on neurodevelopmental assessments at 2 years compared to cases without LD variants, and this difference is similar in isolated and complex cases.

**Conclusion**—We found that the LD genetic variants are associated with higher mortality, worse pulmonary hypertension, and worse neurodevelopment outcomes compared to non-LD variants. Our results have important implications for prognosis, potential intervention and long-term follow up for children with CDH.

## Keywords

Congenital diaphragmatic hernia; De novo mutations; Neurodevelopmental outcome; Pleiotropic; Mortality of birth defects

---

## Introduction

Congenital diaphragmatic hernia (CDH) is a developmental defect of the diaphragm present at birth, occurring in ~3 per 10,000 live births. Improvements in early diagnosis and clinical treatment of CDH have led to improved survival; however, CDH is still associated with significant mortality, long term morbidity, and developmental disabilities in some individuals. Approximately 15–20% of individuals with CDH have an identifiable genetic cause<sup>1–4</sup>, including *de novo* single nucleotide variants (SNVs) and small insertions or deletions (indels) that affect a single gene<sup>5,6</sup>, as well as *de novo* copy number variants (CNVs) that delete or duplicate contiguous genes<sup>7,8</sup>. Pulmonary hypertension (PH) resulting in significant morbidity and mortality and/or developmental delay are more common in CDH patients<sup>9,10</sup>. To date, no studies have comprehensively examined the association of genetic variants with clinical outcomes in CDH patients.

Our previous studies in congenital heart disease (CHD), a common comorbid condition with CDH, have shown increased burden of *de novo* variants in patients with neurodevelopmental disorders<sup>11,12</sup>. We hypothesize that damaging genetic variants that cause congenital anomalies in general often have pleiotropic effect in development and homeostasis of other organs, leading to worse clinical outcomes.

To assess whether *de novo* predicted deleterious variants are associated with differential clinical outcomes in CDH patients, we analyzed 462 CDH patients with parent-offspring trio sequencing enrolled at birth and prospectively followed by the DHREAMS study (Diaphragmatic Hernia Research & Exploration; Advancing Molecular Science). We identified and classified *de novo* SNV, indels, and CNVs, and hypothesized that *de novo* predicted deleterious variants would be associated with higher mortality and worse clinical and developmental outcomes.

## Methods

### Study subjects

The participants in this study were enrolled as neonates with a radiologically confirmed diaphragm defect requiring neonatal repair in the DHREAMS study (Diaphragmatic Hernia Research & Exploration; Advancing Molecular Science; <http://www.cdhgenetics.com>) from 2005 to 2018 and were followed prospectively. DHREAMS is a multi-center study of individuals with a diaphragm anomaly<sup>5</sup>. Columbia University Irving Medical Center (CUIMC) is the central coordinating center and data and biospecimen repository<sup>10,13</sup>. Each enrolling clinical site is a regional tertiary care hospital with a neonatal intensive care unit that includes an extracorporeal membrane oxygenation (ECMO) program<sup>13</sup>. There is no treatment protocol in the study, and clinical management is determined by the treating clinical team. A blood or saliva specimen for genetic analysis was collected from the CDH patient and both parents (trio).

### Ethics statement

All studies were approved by the institutional review boards at each participating institution and the CUIMC Institutional Review Board (IRB), and signed informed consent was obtained.

### Clinical factors and outcomes

**Clinical data**—The clinical data collected for this cohort have been previously described<sup>10</sup>. Briefly, clinical data were prospectively collected from medical records and entered into a central Research Electronic Data Capture (REDCap) database<sup>14</sup>. Parental information including race, highest level of education and occupation were collected at the time of study enrollment by a single research coordinator. The Hollingshead's Four Factor Index of social status (HI), a measure of socioeconomic status (SES), was calculated for each household<sup>15</sup>. Participants with only a diaphragm defect were classified as isolated CDH while participants with at least one additional congenital anomaly (e.g. congenital heart defect, central nervous system anomaly, gastrointestinal anomaly, skeletal anomaly, cleft lip/palate, *etc*) were classified as complex CDH. Pulmonary hypoplasia, cardiac displacement and intestinal

herniation were considered to be part of the diaphragm defect sequence and were not considered independent malformations.

**Outcomes**—Data on the child’s current and past health were gathered by parental interview at two years of age by the CUICM research coordinator. Follow up data were collected through January 2019. Echocardiograms to assess pulmonary hypertension were centrally analyzed independently by two cardiologists using a standardized protocol<sup>10</sup> (see Supplementary Notes). For this study, postoperative pulmonary hypertension (PH) at one month or three months was quantified as none, mild, or severe. Mortality or survival status prior to initial discharge was collected through medical record review. Growth measurements at birth and two years of age, including height and head circumference, were collected and converted to z-scores. Standardized developmental assessments, include the Bayley Scales of Infant Development third edition (BSID-III)<sup>16</sup> and the Vineland Adaptive Behavior Scales second edition (VABS-II)<sup>17</sup>, were administered by child psychologists who were assessed for reliability<sup>10</sup>. The BSID-III and VABS-II were completed either in person at a participating center, locally by certified specialists, or by telephone (VABS-II only). Both assessments were completed with interpreters if the family’s native language was not English. The VABS-II and BSID-III main domains in a standardized population have a mean score of 100 and a standard deviation (SD) of 15.

### Identification of *de novo* variants

At the time the data was frozen for this analysis, 462 of all 647 neonatally enrolled probands met criteria and had trio sequencing data (see Supplementary Notes). The calling and annotation of *de novo* SNVs and indels were processed using a pipeline based on GATK Best Practice v3 followed with heuristic hard filters as described in our previous study<sup>6</sup>. All coding SNVs and indels were classified as synonymous, missense, inframe, or likely-gene-disrupting (LGD, which includes frameshift indels, canonical splice site, or nonsense variants). The missense variants were further predicted as deleterious missense variants by MPC score  $\geq 2$  (unpublished data) and phred-scaled CADD<sup>18</sup> v1.3 score  $\geq 20$ , and plausible deleterious missense variants by MPC score between 1 and 2 and CADD score  $\geq 20$ .

*De novo* CNVs were identified using an inhouse pipeline of read depth-based algorithm based on CNVnator<sup>19</sup> v0.3.3 in genome sequencing trios (Figure S1, Supplementary Notes). If a sample had larger number of *de novo* CNV segments, i.e., more than one SD away from sample mean, the CNVs were called by the additional pair-end/split-read (PE/SR) evidence Lumpy<sup>20</sup> v0.2.13 and genotyped by SVtyper<sup>21</sup> v0.1.4. Only the CNVs supported by both RD and PE/SR would be kept for downstream analysis. *De novo* CNVs were called with at least 2 confirmed evidences: (1) normalized read depth, (2) discordant pairs, (3) split reads, (4) the allelic copy ratio in terms of B allele frequency. 22 of 25 (88%) reported *de novo* CNV in CHD patients were confirmed by quantitative PCR (qPCR). We mapped *de novo* CNVs on GENCODE (v29) protein coding genes with at least 1bp in the shared interval. The GENCODE genes were annotated with variant intolerance metric by gnomAD pLI<sup>22</sup>, haploinsufficiency or triplosensitivity metrics from ClinGen genome dosage map<sup>23</sup>.

## Genetic groups of CDH patients

Based on the enrichment of deleterious *de novo* variants in CDH cases compared to controls or background mutation rate in previous studies<sup>6,8</sup>, we defined likely damaging (LD) variants as: (a) *de novo* LGD or deleterious missense variants in genes that are constrained (gnomAD pLI  $\geq 0.9$ ) and highly expressed in developing diaphragm<sup>8</sup>, or (b) *de novo* LGD or deleterious missense variants in known risk genes of CDH or commonly comorbid disorders (CHD), or (c) plausible deleterious missense variants in known risk genes of CDH or commonly comorbid disorders (CHD), or (d) deletions in constrained (gnomAD pLI  $\geq 0.9$ ) or haploinsufficient genes from ClinGen genome dosage map, or (e) CNVs implicated in known syndromes. We classified CDH patients into two genetic groups: (1) LD, if the patient carried at least one LD variant; (2) non-LD, if the patient carried no such variants.

## Statistical analysis of associations of genetic factors with clinical outcomes

We calculated all pairwise Spearman correlations of all clinical data and outcomes of CDH patients. We considered these nine clinical outcomes in the association analysis: mortality prior to initial discharge, pulmonary hypertension at 1 month and 3 months, growth (height and head circumference) at 2 years, BSID-III (language, cognitive, and motor scores) at 2 years, and VABS-II adaptive behavior composite score at 2 years.

**Association tests**—To estimate the difference of outcomes between LD group and non-LD group, we carried out Fisher’s exact test or Student’s t-test for binary or quantitative outcomes as appropriate. Additionally, we used Lasso selected features as covariates to account for other clinical factors that were correlated with outcomes (see Supplementary Notes). The association tests were analyzed separately for all CDH, isolated and complex CDH cases, but with same covariates for each outcome.

**Independent tests definition**—Since our primary outcomes were highly correlated (Figure S5), the burden of multiple test adjustment was smaller than the number of tests. We took an approach similar to Werling’s method<sup>24,25</sup> to estimate the number of independent tests for multiple-test adjustment. Specifically, we performed eigenvalue decomposition on correlation matrix of all the outcomes used for association with genetic factors and counted the number of principle components ( $N$ ) that combined explain 99% of the variance of the original data. We then set the Bonferroni correction threshold at  $0.05/N$ .

**Software**—Statistical and graphical analyses were performed using R version 3.4.1, with R packages “glmnet”, “pcaMethods”, “sjstats”, “dplyr”, “corrplot”, “ggplot2”, “gridExtra”.

## Results

### Sample characteristics

A total of 647 of approximately 840 eligible neonates born at participating centers during the study time period consented to enrollment in the DHREAMS study (Figure 1). Of 462 patients who had genetic data, 428 cases had exome and/or genome sequencing, including 125 cases with exome sequencing, 394 cases with genome sequencing and 91 cases with both exome and genome sequencing. Thirty cases had conditions diagnosed first through

clinical testing (6 of 30 also had exome sequencing), 4 cases were finished cytogenetic and microarray (CMA)<sup>5</sup> and 7 cases with candidate gene test (1 of 7 also had exome sequencing) (Table S1). The other 185 patients did not have DNA on both biological parents or had insufficient DNA on the proband and had the same clinical characteristics as the 647 cases and 462 cases with genetic data (Table S2). Some participants included in this series have been previously published including CNV analyses<sup>5</sup>, 52 trios with exome sequencing and 117 trios with genome sequencing<sup>6</sup>. The LD group was composed of 85 participants including 43 with *de novo* LGD or deleterious missense variants in known risk genes or constrained genes with high diaphragm expression, 9 with plausible deleterious missense variants in known risk genes (Table S3), 7 with deletions in constrained or haploinsufficient genes (Table S4), 4 with CMA large deletions<sup>5</sup> and 29 genetically diagnosed subjects through clinical genetic testing. The non-LD group was composed of the other 377 participants. As reported in our previous SNV and indels analyses in CDH cases<sup>6</sup> and CNV calls analyses in this study (Figure S3), there were no technical batch effects and the sequencing depth did not affect the variants calling.

The participant's demographic and clinical information is summarized in Table 1. Two thirds of the participants (67%) were white, non-Latino, and slightly more than half (57%) were male. The diaphragm lesion was left sided for 80%, and the diaphragm defect was prenatally diagnosed for 75%. On average, participants were full term (mean 38-week gestation) with an average Apgar of 5 at 1 minute and 7 at 5 minutes. A total of 229 (35%) cases had additional congenital anomalies and were defined as complex; 394 (61%) participants had only a diaphragm defect without additional anomalies and were defined as isolated and the complexity status was unknown for 24 (4%). One third (32%) were treated with extracorporeal membrane oxygenation (ECMO) and over half (52%) received inhaled nitric oxide. Of the 566 participants who survived to surgical repair, 54% required a patch to repair the diaphragm. Two thirds required oxygen therapy at 28 days of life. Pulmonary hypertension was present for 56% of the 399 participants who were assessed at 1 month and 20% of the 306 participants who were assessed at 3 months. The cohort had 22% (144/647) mortality prior to discharge; 23 participants were still hospitalized at the time of the data freeze (1/1/2019) so discharge status was unknown. Six participants died between initial discharge and 2 years of age, and three participants died between 2 and 5 years of age.

At the time of data analysis, 275 of the 385 (71%) participants who had reached 2 years of age had a two-year developmental assessment (Figure S4, BSID-III n=222, VABS-II n=274). The average age at assessment was 25 months with a range of 22 to 36 months (one outlier assessment completed at 14 months). On average, the BSID-III scores for all domains and VABS-II composite scores were 6–7 points below the normal mean of 100. Growth outcomes (standardized z-scores of height and head circumference) at two years of age were completed on 249 participants. On average, the height at 2 years was 0.24 below the normal mean z-scores of 0 and was similar (0.02) to normal head circumference mean at 2 years. As expected, all measures were highly inter-correlated (Figure S5). There were totally 27 tests considering the association analyzed in all cases, isolated cases and complex cases separately. We took eigenvalue decomposition to estimate 10 effective tests based on the sum of eigenvalues that explain 99% of variation (Figure S6). The significant threshold was  $5 \times 10^{-3}$  correcting for multiple tests.

### Higher mortality rate in complex CDH cases and CDH cases with LD variants

Of 462 CDH cases analyzed by genetic status, 31% (53/170) of complex cases and 11% (32/291) of isolated cases carried LD variants (Table S5). The overall mortality in this group was 20% before initial discharge. Complex cases had a higher mortality rate than isolated cases (31% vs. 13%,  $P=8\times 10^{-6}$ ). Compared to the non-LD group, mortality prior to initial discharge was higher in the LD group ( $P=4\times 10^{-4}$ , Figure 2A). The difference in mortality between LD and non-LD group was largely attributable to differences in the isolated cases. The isolated cases with LD variants had a mortality rate (31%) which was similar to complex cases and was much higher than isolated cases without LD (11%,  $P=3\times 10^{-3}$ ).

### Higher PH prevalence in CDH cases with LD variants

A total of 335 cases with genetic data were assessed for PH at 1 month. Overall 178/335 of the cases with genetic data (53%) were diagnosed with PH at 1 month. Complex cases had a trend of higher frequency of PH at 1 month than isolated cases (62% vs. 48%,  $P=1\times 10^{-2}$ , Figure 2B). LD cases had significantly higher prevalence of PH at 1 month than non-LD cases (74% vs. 49%,  $P=8\times 10^{-4}$ ). Similar to mortality, the difference was largely driven by isolated cases: isolated cases with LD had a frequency similar to complex cases and higher than isolated cases without LD (85% vs. 44%,  $P=1\times 10^{-3}$ ). The overall prevalence of PH at 3 months was much lower (51/268=19%), and had a higher prevalence in cases with LD variants remained (37% vs. 16%,  $P=3\times 10^{-3}$ , Figure 2C).

### LD variants were associated with worse neurodevelopmental status at two years of age

LD cases had significantly lower BSID-III language ( $\beta=17$ ,  $P=5\times 10^{-5}$ ), BSID-III cognition ( $\beta=14$ ,  $P=1\times 10^{-4}$ ), BSID-III motor ( $\beta=15$ ,  $P=3\times 10^{-4}$ ), and VABS-II adaptive behavior composite ( $\beta=12$ ,  $P=4\times 10^{-5}$ ) scores than non-LD cases (Figure 3). There was a consistent trend of the pattern in isolated and complex cases. Adaptive Behavior function from the VABS-II had the most significantly different (mean score 84 vs. 96,  $\beta=12$ ,  $P=4\times 10^{-5}$ ), consistent across isolated ( $\beta=11$ ) and complex cases ( $\beta=10$ ). For all neurodevelopmental domains in both the BSID-III and VABS-II, the complex cases had worse neurodevelopmental outcomes than isolated cases in the corresponding genetic groups (Figure 3).

Our previous studies<sup>9</sup> showed that many different clinical factors were associated with developmental delay. To account for the factors that had independent associations with outcomes, we used Lasso regularized regression to select these factors and then included them as covariates in linear regression models to test the association of LD variants with developmental outcomes. By penalizing the absolute size of the regression coefficients, the Lasso regression ends up in a situation where some of the parameter estimates may be exactly zero, which is convenient when the predictors are highly correlated<sup>26,27</sup> (Figure S5). With Lasso-selected clinical factors as covariates, we estimated the effect size of LD variants to BSID-III and VABS-II scores. We found that the effect size of LD variants was similar to the estimates from the univariate test without clinical factors as covariates for all measures except BSID-III language. Specifically, with BSID-III language, there was still a trend toward worse score in LD patients ( $\beta=8.5$ ) without reaching statistical significance after correcting multiple tests ( $P=9\times 10^{-3}$ ). Notably, families with one more point of

socioeconomic status normalized z score based on parental education and occupation had 4.5 points increased scores on BSID-III language domain ( $\beta=4.3$ , 95% CI=2.2–6.4,  $P=1\times 10^{-4}$ ), and oxygen at discharge had substantial adverse impact on BSID-III language domain scores ( $\beta=12$ , 95% CI=5.5–18,  $P=3\times 10^{-4}$ ), consistently in both isolated and complex cases. With BSID-III cognitive score, LD patients had significantly worse outcome than non-LD patients ( $\beta=11.4$ , 95% CI=5.7–17,  $P=1\times 10^{-4}$ ). The difference was more pronounced in complex cases ( $\beta=16.6$ , 95% CI=7.5–25.8,  $P=8\times 10^{-4}$ ), whereas the effect size was small in isolated cases ( $\beta=3.4$ , 95% CI=-11.5–4.8,  $P=0.4$ ). BSID-III cognitive domain scores also had a similar trend that associated with SOI (oxygen at discharge:  $\beta=7.0$ , 95% CI=1.1–12.8,  $P=2\times 10^{-2}$ ; and ECMO:  $\beta=5.4$ , 95% CI=0.4–10.5,  $P=4\times 10^{-2}$ ). With BSID-III motor domain scores, LD patients had significantly lower scores ( $\beta=14.7$ , 95% CI=9.1–20.3,  $P=8\times 10^{-7}$ ), which was consistent in isolated ( $\beta=15.5$ , 95% CI=7.3–23.7,  $P=3\times 10^{-4}$ ) and had a similar trend in complex cases ( $\beta=10.6$ , 95% CI=2.0–19.3,  $P=2\times 10^{-2}$ ). BSID-III motor domain scores were significantly associated with SOI (oxygen at discharge:  $\beta=12.8$ , 95% CI=6.9–18.7,  $P=3\times 10^{-5}$ ), and the effect was consistent in both isolated and complex cases ( $\beta=15$  and 11, respectively). With VABS-II composite adaptive behavior score, patients with LD variants had significantly lower scores ( $\beta=10.7$ , 95% CI=6.5–14.8,  $P=1\times 10^{-6}$ ), consistently in both isolated and complex cases ( $\beta=10.2$  and 8.6, respectively).

LD cases was lower ( $\beta=0.86$  for z-score,  $P=1\times 10^{-3}$ , Figure S7) than non-LD cases at 2 years of age. There was no significant difference with head circumference at 2 years of age (Figures S7–S9).

After the exclusion of carriers with known syndromic abnormalities and/or large CNVs (Table S1), the association of worse clinical outcomes for LD cases compared to non-LD cases were consistent with similar effect size (Figures S9–11, see Supplementary Notes). Lastly, there was no difference in clinical outcomes and factors between clinical sites ( $P>0.05$ ), which was consistent with our prior study of the DHREAMS cohort<sup>10</sup>. There was no statistical difference was observed in mortality, need for ECMO, and percentage with pulmonary hypertension at one month of age across sites.

## Discussion

To our knowledge, this is the first study assessing the association between genetic variants and outcomes at two year of age in CDH patients. We classified 462 CDH neonates into two genetic groups: (1) CDH patients with likely damaging variants, including *de novo* SNVs, indels, CNVs involving known risk genes or constrained genes with high diaphragm expression and (2) other CDH patients. We assessed the association of these genetic classes with mortality prior to initial discharge, pulmonary hypertension, neurodevelopmental outcomes and growth measurements at two years of age for genetic groups. We found that *de novo* likely damaging variants were associated with higher mortality and prevalence of PH, worse neurodevelopmental outcomes at two years of age, but no difference in height or head circumference at two years. The associations were largely observed in both complex cases and isolated cases, and independent of other clinical factors.



The underlying hypothesis of this study is that damaging variants that cause CDH often have pleiotropic effects on the development of other parts of the body, leading to overall worse clinical and neurodevelopmental outcomes. One challenge in testing this hypothesis in this early stage of understanding CDH genetics is how to identify damaging variants when established risk genes contribute to only a small fraction of cases<sup>1,6</sup>. Previous genetic studies of CDH<sup>6–8,28</sup> and a common comorbid condition CHD<sup>11,12</sup> showed that cases are enriched with deleterious *de novo* variants, especially in genes that are highly expressed in the affected organ during development<sup>8,11</sup>, or in genes that are intolerant of variants in general population<sup>6</sup>. Collectively, about 10–30% of CDH cases are attributable to *de novo* variants. Based on these observations, we defined likely damaging variants as deleterious *de novo* variants in established risk genes or genes that are constrained and highly expressed in developing diaphragm. By this approach, we identified likely damaging variants in about 20% of CDH cases. As we develop better methods to identify genes relevant to CDH development, we should be able to identify additional monogenic and oligogenic *de novo* and inherited variants contributing to CDH and clinical outcomes.

In our study, cases with LD variants had worse clinical and neurodevelopmental outcomes than cases without LD variants. Additionally, complex cases generally had worse outcomes than isolated cases. The effect of LD variants was consistent in both isolated and complex cases with a few exceptions, indicating that LD variants affect clinical outcome independent of the presence of additional anomalies at birth.

Previous studies indicated that abnormal pulmonary vascular development and function is a significant clinical problem in CDH infants<sup>29</sup>. In our cohort, CDH cases with LD variants had higher PH prevalence at 1 or 3 months compared to cases without LD variants. The mechanism of PH in CDH could be in part the pleiotropic effect of genes with LD variants that lead to the premature differentiation of smooth muscle cells into a contractile phenotype<sup>30</sup>. The PH prevalence is lower at 3 months due to early mortality in those severely affected and due to continued maturation of the pulmonary vasculature and efficacy of clinical treatments.

The neurodevelopmental scores in the BSID-III and VABS-II were assessed for correlation, especially for the similar domains. Patients with LD variants had worse motor and cognitive function, likely reflecting effects of these genes on overall brain function. The VABS-II assesses adaptive behaviors including communication, daily living skills, socialization and motor skills, and LD patients had worse adaptive behavioral skills compared to non-LD patients. BSID-III language is correlated with SES, and this was also the case in our study. SOI, i.e., oxygen at discharge, is likely a measure of overall clinical status and likely reflects disease severity which may be inversely correlated with the child's opportunity to develop new skills. It is important to note that even though this is a multicenter study and clinical management was left to the discretion of each center's clinical teams.

## Limitations

While we report the largest prospective study of the natural history, genetic etiology and clinical outcomes in patients with CDH, there are several limitations to our study. We did not successfully recruit all eligible participants. Our experience is that the most severe cases

who died shortly after birth were less likely to enroll. Not all probands had DNA available on both parents, and therefore were not analyzed in this study although we did not observe significant differences in clinical characteristics for that group. Some patients did not complete all prospective clinical assessments, in particular the neurodevelopmental assessment, and there may be some unknown bias in what families completed those assessments. Our study is limited by the relatively young age at assessment, as some children may present with learning and/or behavioral disabilities as they get older.

Since our knowledge of CDH risk genes is incomplete and the methods to interpret deleterious missense variants are imperfect, it is likely that the groups of cases with LD or non-LD variants are not precise. Future gene discovery studies with larger sample sizes will allow us to identify damaging variants with greater accuracy and reassess the association of outcomes.

In summary, our association analysis demonstrates that CDH cases with *de novo* LD variants had higher mortality prior to discharge, higher prevalence of pulmonary hypertension and worse neurodevelopmental outcomes at 2 years of age. Accurate prognostic information is critical for prenatal diagnosis and counseling and neonatal management to help the families and clinicians make informed decisions about management.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

We would like to thank the patients and their families for their generous contribution. We are grateful for the technical assistance provided by Patricia Lanzano, Jianguan Hu, Jiancheng Guo, Liyong Deng, Donna Garey, and Ankeil Abreu from Columbia University. We thank our clinical coordinators and developmental specialists across the DHREAMS centers: Karen Lukas, Desiree White, and Jessica Conway at Washington University School of Medicine, Patricia Burns, Melissa Spare, and Sarah (Lexie) Price at Cincinnati Children's Hospital, Sheila Horak and Kelly Miller at Children's Hospital & Medical Center of Omaha, Jeannie Kreutzman and Jennifer Butcher at CS Mott Children's Hospital, Tracy Perry at Monroe Carell Jr. Children's Hospital, Michelle Kallis at Northwell Health, Brandy Gonzales and Alicia McIntire at Oregon Health and Science University, Gentry Wools and Lorrie Burkhalter at Children's Medical Center Dallas, Elizabeth Jehle at Hassenfeld Children's Hospital at NYU Langone Health, Michelle Knezevich and Cheryl Kornberg at Medical College of Wisconsin, Min Shi at Children's Hospital of Pittsburgh.

### Funding

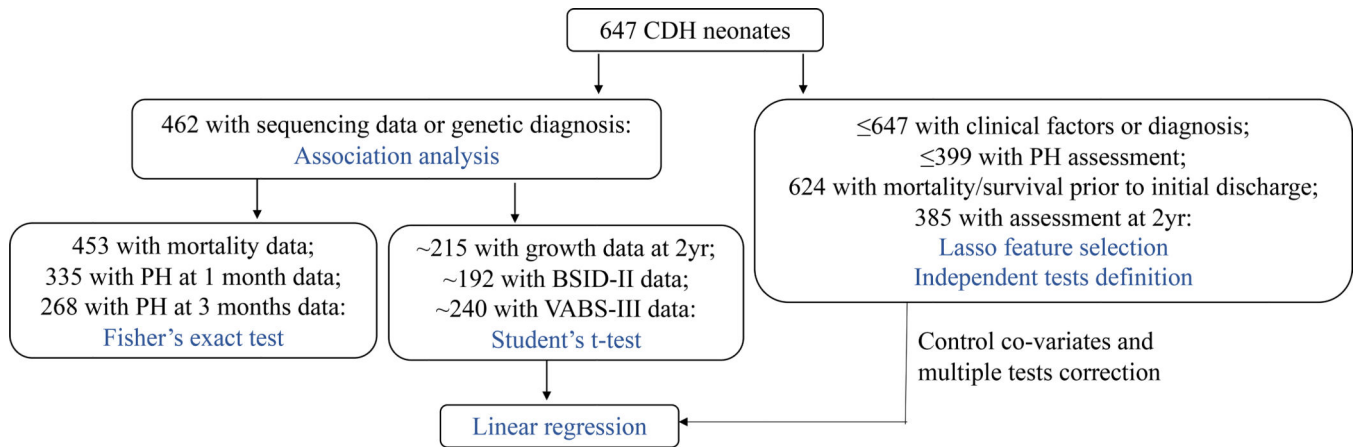
The genome sequencing data were generated through NIH Gabriella Miller Kids First Pediatric Research Program (X01HL132366, X01HL136998, and X01HL140543) and the University of Washington Center for Mendelian Genomics with NHGRI grant HG006493 (D.N., M.B., S.L.). This work was supported by NIH grants R01HD057036 (L.Y., J.W., W.K.C.), R03HL138352 (A.K., W.K.C., Y.S.), R01GM120609 (H.Q., Y.S.), UL1 RR024156 (W.K.C.), and 1P01HD068250 (P.K.D., M.L., F.A.H., J.M.W., W.K.C., Y.S.). Additional funding support was provided by grants from CHERUBS, CDHUK, and the National Greek Orthodox Ladies Philoptochos Society, Inc. and generous donations from Fore Hadley, the Williams Family, Wheeler Foundation, Vanech Family Foundation, Larsen Family, Wilke Family and many other families. Shannon Nees received salary support through a Ruth L. Kirschstein National Research Service Award of the NIH under award number 5T32HL007854-22. The genome sequencing data can be obtained from dbGAP through accession phs001110.

## References

1. Yu L, Hernan RR, Wynn J, Chung WK. The influence of genetics in congenital diaphragmatic hernia. *Seminars in perinatology*. 2019;151169. [PubMed: 31443905]

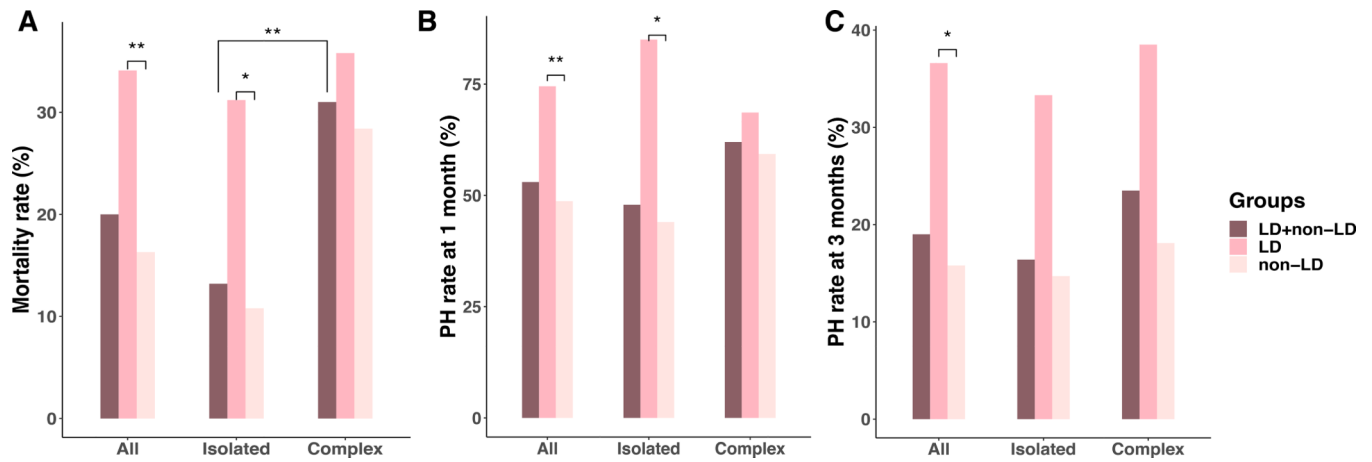
2. Kammoun M, Souche E, Brady P, et al. Genetic profile of isolated congenital diaphragmatic hernia revealed by targeted next-generation sequencing. *Prenat Diagn.* 2018;38(9):654–663. [PubMed: 29966037]
3. Slavotinek AM. The genetics of common disorders - congenital diaphragmatic hernia. *Eur J Med Genet.* 2014;57(8):418–423. [PubMed: 24793812]
4. Pober BR, Lin A, Russell M, et al. Infants with Bochdalek diaphragmatic hernia: sibling precurrence and monozygotic twin discordance in a hospital-based malformation surveillance program. *Am J Med Genet A.* 2005;138A(2):81–88. [PubMed: 16094667]
5. Yu L, Wynn J, Ma L, et al. De novo copy number variants are associated with congenital diaphragmatic hernia. *J Med Genet.* 2012;49(10):650–659. [PubMed: 23054247]
6. Qi H, Yu L, Zhou X, et al. De novo variants in congenital diaphragmatic hernia identify MYRF as a new syndrome and reveal genetic overlaps with other developmental disorders. *PLoS genetics.* 2018;14(12):e1007822. [PubMed: 30532227]
7. Zhu Q, High FA, Zhang C, et al. Systematic analysis of copy number variation associated with congenital diaphragmatic hernia. *Proceedings of the National Academy of Sciences of the United States of America.* 2018;115(20):5247–5252. [PubMed: 29712845]
8. Yu L, Sawle AD, Wynn J, et al. Increased burden of de novo predicted deleterious variants in complex congenital diaphragmatic hernia. *Hum Mol Genet.* 2015;24(16):4764–4773. [PubMed: 26034137]
9. Wynn J, Aspelund G, Zygmunt A, et al. Developmental outcomes of children with congenital diaphragmatic hernia: a multicenter prospective study. *J Pediatr Surg.* 2013;48(10):1995–2004. [PubMed: 24094947]
10. Wynn J, Krishnan U, Aspelund G, et al. Outcomes of congenital diaphragmatic hernia in the modern era of management. *J Pediatr.* 2013;163(1):114–119 e111. [PubMed: 23375362]
11. Homsy J, Zaidi S, Shen Y, et al. De novo mutations in congenital heart disease with neurodevelopmental and other congenital anomalies. *Science.* 2015;350(6265):1262–1266. [PubMed: 26785492]
12. Jin SC, Homsy J, Zaidi S, et al. Contribution of rare inherited and de novo variants in 2,871 congenital heart disease probands. *Nat Genet.* 2017;49(11):1593–1601. [PubMed: 28991257]
13. Farkouh-Karoleski C, Najaf T, Wynn J, et al. A definition of gentle ventilation in congenital diaphragmatic hernia: a survey of neonatologists and pediatric surgeons. *J Perinat Med.* 2017;45(9):1031–1038. [PubMed: 28130958]
14. Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap)—a metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform.* 2009;42(2):377–381. [PubMed: 18929686]
15. Hollingshead A Four Factor Index of Social Status. 2011; *Yale Journal of Sociology*(8):21–51.
16. Bayley N Bayley Scales of Infant and Toddler Development-Third Edition. TX: Harcourt Assessments, Inc, San Antonio 2006.
17. Harrison PL, Oakland T. Adaptive behavior assessment system (2nd ed.). San Antonio: Harcourt 2003.
18. Rentzsch P, Witten D, Cooper GM, Shendure J, Kircher M. CADD: predicting the deleteriousness of variants throughout the human genome. *Nucleic Acids Res.* 2019;47(D1):D886–D894. [PubMed: 30371827]
19. Abyzov A, Urban AE, Snyder M, Gerstein M. CNVnator: an approach to discover, genotype, and characterize typical and atypical CNVs from family and population genome sequencing. *Genome Res.* 2011;21(6):974–984. [PubMed: 21324876]
20. Layer RM, Chiang C, Quinlan AR, Hall IM. LUMPY: a probabilistic framework for structural variant discovery. *Genome Biol.* 2014;15(6):R84. [PubMed: 24970577]
21. Chiang C, Layer RM, Faust GG, et al. SpeedSeq: ultra-fast personal genome analysis and interpretation. *Nat Methods.* 2015;12(10):966–968. [PubMed: 26258291]
22. Samocha KE, Robinson EB, Sanders SJ, et al. A framework for the interpretation of de novo mutation in human disease. *Nat Genet.* 2014;46(9):944–950. [PubMed: 25086666]
23. Riggs ER, Church DM, Hanson K, et al. Towards an evidence-based process for the clinical interpretation of copy number variation. *Clin Genet.* 2012;81(5):403–412. [PubMed: 22097934]

24. Werling DM, Brand H, An JY, et al. An analytical framework for whole-genome sequence association studies and its implications for autism spectrum disorder. *Nat Genet.* 2018;50(5):727–736. [PubMed: 29700473]
25. Nyholt DR. A simple correction for multiple testing for single-nucleotide polymorphisms in linkage disequilibrium with each other. *Am J Hum Genet.* 2004;74(4):765–769. [PubMed: 14997420]
26. Santosa F, Symes WW. Linear Inversion of Band-Limited Reflection Seismograms. *SIAM Journal on Scientific and Statistical Computing.* 1986;7(4):1307–1330.
27. Tibshirani R Regression Shrinkage and Selection via the Lasso. *Journal of the Royal Statistical Society.* 1996;58(1):267–288.
28. Longoni M, High FA, Qi H, et al. Genome-wide enrichment of damaging de novo variants in patients with isolated and complex congenital diaphragmatic hernia. *Hum Genet.* 2017;136(6):679–691. [PubMed: 28303347]
29. Mous DS, Kool HM, Wijnen R, Tibboel D, Rottier RJ. Pulmonary vascular development in congenital diaphragmatic hernia. *Eur Respir Rev.* 2018;27(147).
30. Sluiter I, van der Horst I, van der Voorn P, et al. Premature differentiation of vascular smooth muscle cells in human congenital diaphragmatic hernia. *Exp Mol Pathol.* 2013;94(1):195–202. [PubMed: 23018129]

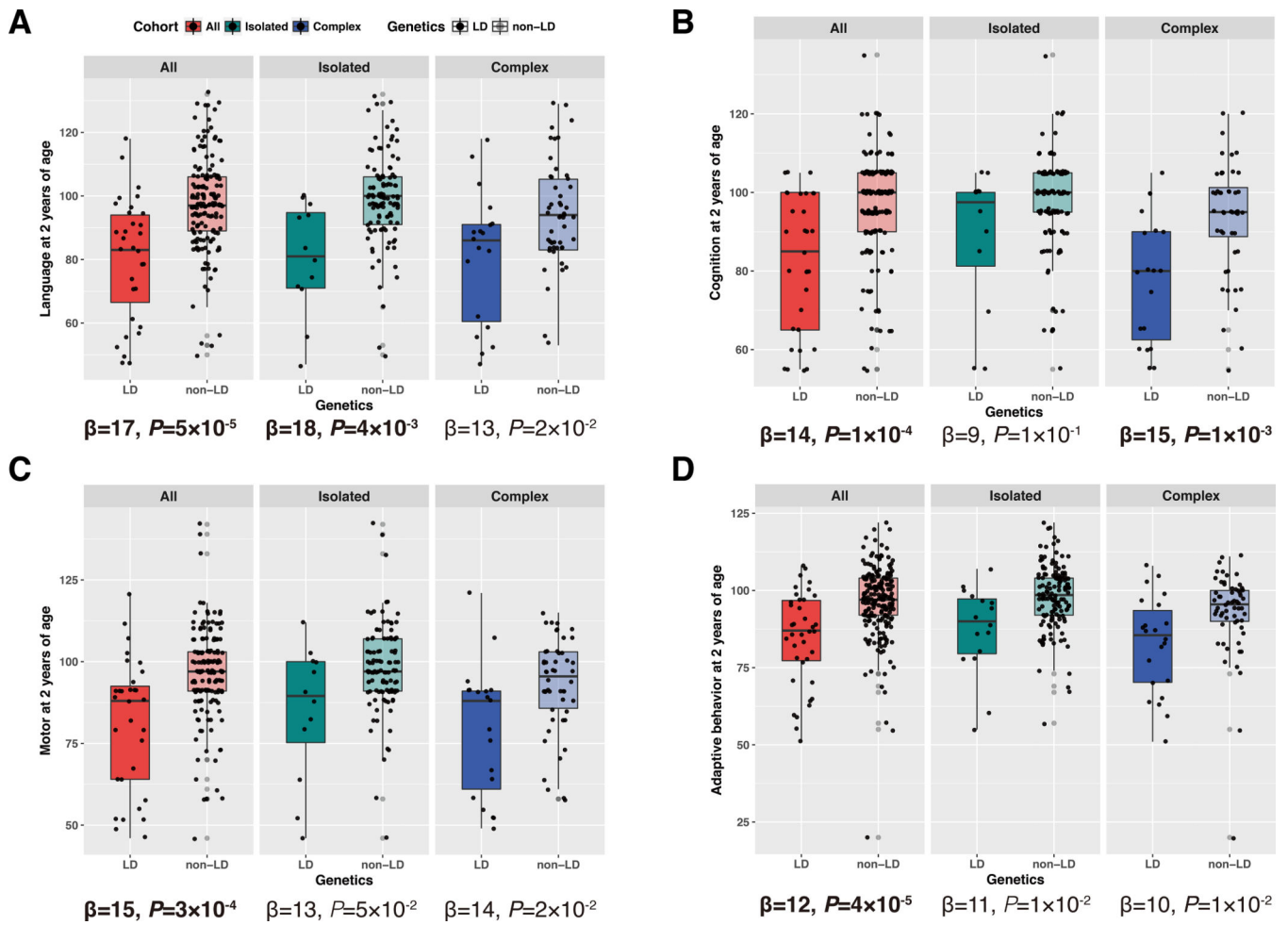


**Figure 1. Study design and number of patients.**

PH: pulmonary hypertension; 2yr: 2 years of age; BSID-III: Bayley Scales of Infant Development third edition; VABS-II: Vineland Adaptive Behavior Scales second edition.

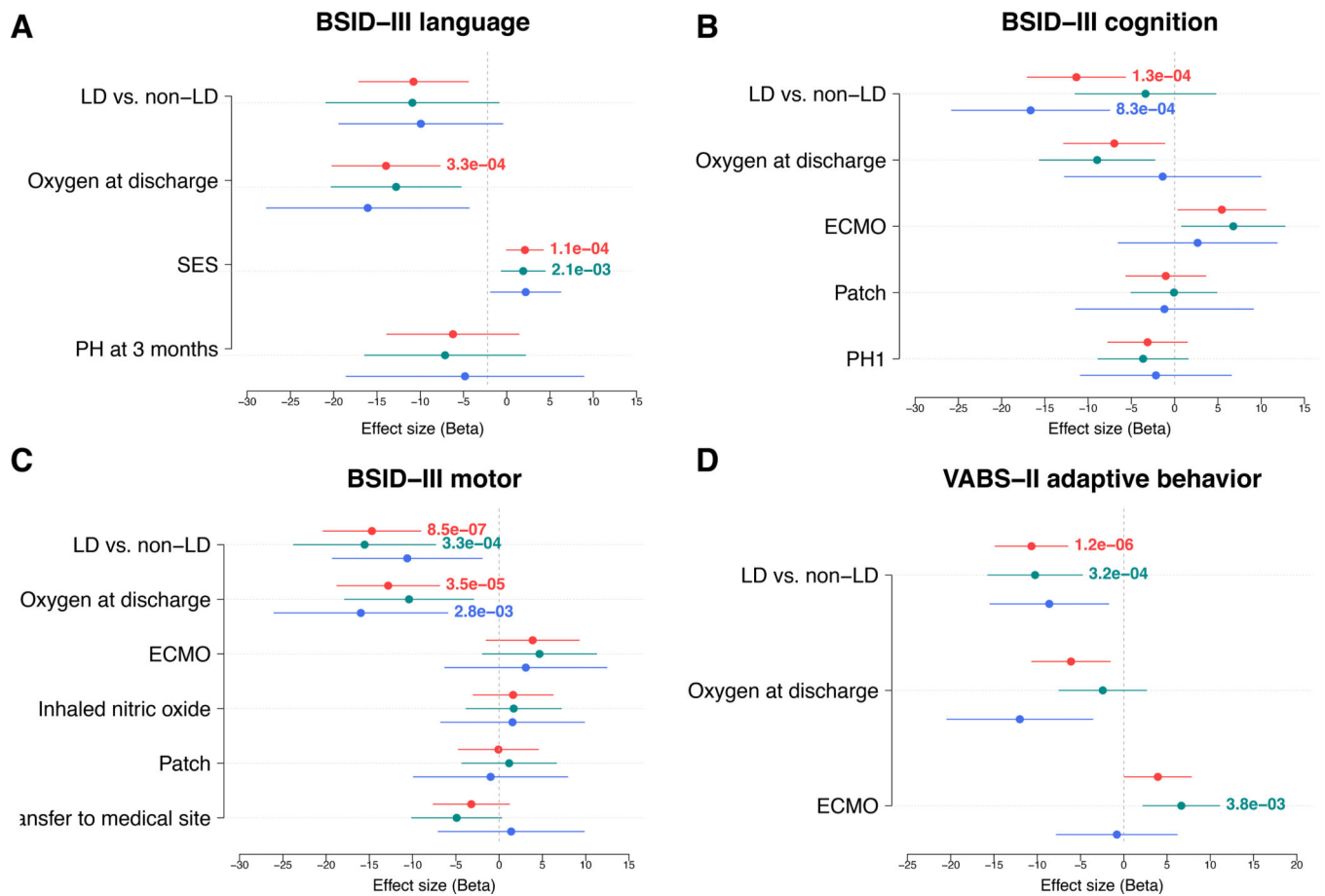


**Figure 2. Association analyses for genetic groups with mortality and pulmonary hypertension.** (A) Mortality prior to initial discharge. (B) Pulmonary hypertension at 1 month. (C) Pulmonary hypertension at 3 months. *P* value is given by Fisher's exact test after correcting multiple tests ( $P < 5 \times 10^{-3}$ ). \*\*:  $P < 0.001$ ; \*:  $P < 0.005$ .



**Figure 3. Association analyses for genetic groups with neurodevelopmental outcomes at 2 years of age.**

(A) BSID-III language, (B) BSID-III cognition, (C) BSID-III motor, (D) VABS-II composite adaptive behavior. The black line in the middle of the box is the mean value for each group. The vertical size of the box is the interquartile range (IQR). The whisker is 1.5xIQR. *P* value is given by Student's *t*-test with bold as significant after correcting multiple tests ( $P < 5 \times 10^{-3}$ ).



**Figure 4. Effect size for likely damaging variants on neurodevelopmental outcomes at 2yr comparing to non-LD variants.** (A) BSID-III language, (B) BSID-III cognitive, (C) BSID-III motor, (D) VABS-II composite adaptive behavior. Effect size is the coefficient/beta, which aligns with outcome on the y-axis, with the vertical dashed line ( $v=0$ ) separating positive and negative direction of association. Significant  $P$  value after correcting multiple tests ( $P < 5 \times 10^{-3}$ ) is noted. LD: likely damaging variants; SES: socioeconomic status; PH: pulmonary hypertension; ECMO: extracorporeal membrane oxygenation.



**Table 1.**

Clinical characteristics of CDH patients

	All patients (n=647)		Patients with genetic data (n=462)	
	Number	Percent/Mean(SD)	Number	Percent/Mean(SD)
<b>Neonatal Factors</b>				
Parental Hollinghead Score (SES)	422	41.9 (12.9)	352	42.7 (13.0)
Father College or Greater	511	35.6%	406	39.2%
Mother College or Greater	524	42.8%	409	47.4%
Family Annual Income < \$30,000	501	36.3%	393	30.3%
White and non-Hispanic race	555	75.1%	444	78.4%
Female sex	638	42.3%	462	43.5%
Inborn in enrolling clinical site	608	58.6%	449	61.2%
Prenatal diagnosis	621	74.5%	457	75.5%
Gestational age, weeks	623	37.7 (2.0)	460	37.7 (2.0)
Birth weight, z-score	605	-0.32 (1.1)	453	-0.26 (1.1)
Birth length, z-score	567	0.30 (1.4)	428	0.33 (1.4)
Birth head circumference, z-score	541	0.39 (1.4)	405	0.45 (1.4)
Apgar 1 minute	604	4.93 (2.5)	446	5.05 (2.5)
Apgar 5 minute	601	6.93 (2.1)	443	7.07 (2.0)
<b>Diagnosis</b>				
Left lesion side	629	82.3%	462	82.9%
Additional congenital anomalies (complex CDH)	623	36.8%	461	36.9%
PH at 1 month	399	55.6%	335	53.1%
PH at 3 months	306	19.6%	268	19.0%
<b>Treatment</b>				
Diaphragm repaired with patch	566	53.7%	432	61.3%
Extracorporeal Membrane Oxygenation (ECMO)	618	31.5%	455	30.1%
Inhaled nitric oxide	599	52.2%	450	53.1%
Tracheostomy	587	4.4%	443	3.8%
Oxygen required at 28 days	481	68.8%	279	63.4%
Oxygen required at discharge	77	16.1%	369	16.3%
Tube feeding at discharge	474	49.2%	367	49.6%